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GA4 AND tZR QUANTIFICATION IN PISTILLATE AND STAMINATE PLANTS OF *DASYLIRION CEDROSANUM*

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Abstract

'Sotol', a protected designation of origin alcoholic beverage, is obtained from *Dasylirion cedrosanum* spp. plants. There is little knowledge concerning the sexual differentiation mechanisms of this species, which leads to a lack of proposals for not only its conservation and reforestation but also mechanisms to account for the dioecious nature of the plant. Phytohormones have been associated with sexual differentiation in dioecious plants because, individually or in combination, these hormones promote masculinization or feminization of their sexual structures. The objective of this study was to quantify gibberellin A4 (GA4) and trans-zeatin riboside (tZR) levels in samples of different organs of staminate and pistillate plants of *D. cedrosanum*, which were collected at different stages of floral development. The plant material was obtained at three locations in Coahuila at North Mexico. Gibberellin A4 (GA4) and trans-zeatin riboside (tZR) were quantified by HPLC-UV at 205 nm and 268 nm, respectively. During the later appearance of pollen and seeds, the GA4 levels in the crown and leaves were the same but exceeded those in the inflorescence. There were no differences in tZR levels between the plants of different sexes. Among organs, differences were only found during inflorescence emergence and death, stages during which the crown presented the highest levels of tZR. The results for the GA4/tZR ratio were similar to those reported for GA4. GA4 most likely plays a role in sexual determination in *D. cedrosanum* because its presence is associated with the appearance of staminate flowers.

Key words: Cytokinins, Gibberellins, Phytohormones, Sexual differentiation, Sotol.

Introduction

Most flowering plants in the world are hermaphroditic. However, a minority of hermaphroditic flowering plants evolved toward two different sexual forms, that is, toward dioecy. Approximately three-quarters of the flowering plant families include dioecious plant species. Dioecy occurs in the *Asparagaceae* family but is not especially prevalent (Ainsworth, 2000).

Dasylirion cedrosanum is a dioecious plant belonging to the *Asparagaceae* family, Nolinaceae subfamily. This subfamily is distributed in Northern Mexico and the Southwestern United States at elevations of 950-2000 meters above sea level (MASL) (Bogler, 1994). Certain species of the genus, such as *D. duranguense*, *D. wheeleri*, and *D. cedrosanum*, have economic significance because they are used regardless of their sex to obtain extracts with high sugar content. Those extracts are obtained from ground, boiled, and fermented crown tissues and are used to create an alcoholic beverage common in regions where the plant grows (De la Garza, 2008). Local populations refer to this beverage by several names, such as "sotol," "zotol", or "sereque." In 2002, "Sotol," as it is known in Mexico (Anon., 2004), obtained a protected designation of origin. For the inhabitants of the arid regions where different species of *Dasylirion* are found, the artisanal production of the sotol beverage is one of the most significant sources of income. Nevertheless, little information concerning the biology of these plants is available, especially concerning their reproductive characteristics. This lack of knowledge has contributed to the poor management of natural populations and an overexploitation of this natural resource. This overexploitation could be alleviated by establishing nursery specimens of *D. cedrosanum* to be used in reforestation programs. However, this activity requires techniques for the early determination of the sex of plants. Unfortunately, there is currently no information on this topic because of a poor understanding of the factors involved in sex determination in the *Dasylirion* genus.

Certain determinants of sexual differentiation in dioecious plants are based upon the presence of sex-linked genes, homomorphic X and Y chromosomes, or the presence of compounds such as phytohormones that induce the masculinization or feminization of sex organs (Durand & Durand, 1984; Ming *et al*., 2007; Soldatova & Khryanin, 2010). Gibberellins and cytokinins are among the phytohormones that play a key role in sexual differentiation because these hormones induce masculinization and feminization, respectively, in different species, such as *Cucumis sativus* (Yin & Quinn, 1995b), *Mercurialis annua* (Boissay *et al.*, 1996), *Zea mays* L. (Zhao *et al.*, 1999), *Buchloe dactyloides* (Yin & Quinn, 1995a), and *Cannabis sativa* L. (Soldatova & Khryanin, 2010). However, note that sex organ masculinization or feminization is not always induced by these hormones. For example, sex determination in male and female asparagus (*Asparagus officinalis*) flowers depends on endogenous levels of auxin and cytokinins, respectively (Bracale *et al*., 1991).

According to the above studies, sex determination in many species showing either monoecy or dioecy is believed to be driven by more than one of the genetic or biochemical mechanisms mentioned above (Soldatova & Khryanin, 2010). Many related phytohormones regulates

sex differentiation in plants, however, the role of these chemicals in sexual differentiation varies from species to species. For this reason a preliminary study was realized to determine the role GA4 and tZR in sexual differentiation of *Dasylirion cedrosanum*. Because there are no records on the role of plant hormones in the genus *Dasylirion*, we proposed to evaluate GA4 and tZR based on a previous report indicating that Gibberellin A4 (GA4) has the potential to increase production of staminate plants, that trans zeatin (tZ) was related to genes responsible for the feminization, and that the precursor of zeatin, trans zeatin riboside (TZR), has been identified as an inducer of masculinization in *Mercurialis annua* (Louis *et al*., 1990). The objectives of this study were to obtain basic information about the concentration of gibberellin A4 (GA4), trans-zeatin riboside (tZR), and the GA4/tZR ratio in different organs of pistillate and staminate plants of *Dasylirion cedrosanum*, as well as obtaining preliminary data potentially useful for improving plant and nursery management, and species conservation.

Materials and Methods

Sampling sites

General Cepeda, Coahuila, México (GC): Located at 25°22′35" N and 101°28′30" W. The average altitude is 1986 MASL. The climate is temperate, semi-dry with little rain in summer and winter, with an average temperature of 19.92 ºC and annual rainfall of 251.6 mm (INIFAP, Records of Climate in 2014). The soil is a silt loam, calcareous, pH=8.3±0.1, with moderately high content of organic matter. The soils are not saline with an electrical conductivity of 0.173±0.004 mmhos/cm.

San Lorenzo Cayon, Saltillo, Coahuila, México (SLC): Located at 25°20′22′′ N and 100°59′23′′ W. The average altitude is 1942 MASL. The climate is dry. Average annual temperature is 16.92 ºC with annual rainfall of 628.6 mm (INIFAP, Records of Climate in 2014). The soil is a clay loam, calcareous, pH=8.3±0.1, with moderately high content of organic matter. The soils are not saline with an electrical conductivity of 0.134±0.020 mmhos/cm.

Universidad Autónoma Agraria Antonio Narro, Saltillo, Coahuila, México (UAAAN): Located at 25°20′58.0" N and 101°01′41.7′′ W. The average altitude is 1842 MASL. The climate is temperate, semi-dry with little rain in summer and winter, with an average temperature of 16.92 °C and annual rainfall of 628.6 mm (INIFAP, Records of Climate in 2014). The soil is loam, calcareous, pH=8.3±0.1, with moderately low content of organic matter. The soils are not saline with an electrical conductivity of 0.218±0.031 mmhos/cm.

Sample collection, processing, and storage: The analysis of GA4 and tZR levels was conducted in leaf, crown, and inflorescence stem from adult pistillate (female) and staminate (male) plants of *D. cedrosanum*. The plants were sampled at the three locations above mentioned: GC, SLC, and UAAAN. The sampling of crown, leaves, and inflorescence was done using a systematic sampling method on plants with a recently emerged floral inflorescence. The sampling of these organs was performed on four pistillate and four staminate plants, with the same plants sampled repeatedly once a week from the time of inflorescence appearance until the inflorescence was completely dried, providing a total of four consecutive samplings. Samplings 1, 2, 3, and 4 refer to different stages: inflorescence emergence, flower appearance, pollen and seed appearance, and inflorescence death, respectively. To minimize damage to the plants, the tissue samples were obtained using a borer, collecting 2-5 g of fresh tissue from the crown and inflorescence. Care was taken to ensure that the samples were no larger than one-third of the inflorescence diameter. The leaf samples were taken from the base of the two youngest, fully developed leaves near the location of inflorescence emergence. The samples were cut into 2 cm pieces for storage. Each sample was placed in a labeled plastic container and immediately immersed in liquid nitrogen until storage at -80 °C in an ultrafreezer (SANYO model MDF-U53VA). After 12 h of freezing, the water in the samples was completely removed using a lyophilizer (LABCONCO Model 2.5L) with a 0.25 mbar vacuum at -40 °C. Finally, the samples were pulverized in a mortar and stored at room temperature in containers with silica gel and hermetic seals.

Gibberellin A4 and tZR extraction: The extraction of both phytohormones was done as described by Hou *et al*. (2008), with slight modifications of the Bieleski's solution reported by Dobrev & Kamínek, (2002), which has been used widely for extraction of cytokinins and gibberellins. A 50 mg portion of each sample was placed in a microtube, and 1 mL of extraction solution [20% (v/v) methanol diluted in 0.1% (v/v) formic acid] was added. The samples were vortexed (Vortex-Genie 1 Touch Mixer, model SI-0136) for 30 s and sonicated (BRANSON model 1510R-DTH sonicator) for 10 min. The samples were then centrifuged at 12,000 rpm for 10 min at 4°C and incubated for 12 h in a freezer at -20°C. After the 12 h incubation, a second sonication was performed for 10 min, followed by centrifugation for 10 min at 12,000 rpm at 4 °C. Finally, the supernatant was filtered through a 0.45 µm pore diameter membrane and transferred to vials to be injected into a liquid chromatograph.

Gibberellin A4 determination by HPLC: The procedure for the quantification of GA4was modified from the methodology originally described by Aktas *et al*. (2008), using a high-performance liquid chromatograph (VARIAN model 920LC) with a UV detector. The separation and identification of GA4 were performed at room temperature using a POLARIS 5 C18-A column with the following dimensions: 250 mm length, 4.5 mm internal diameter, and 5 µm particle size (VARIAN brand). The mobile phases, containing 100 mM acetic acid and 100% acetonitrile mixed 50:50 (v/v), were used in isocratic mode at 0.8 mL/min flux. The plant extract injection volume was 50 µL, and molecule detection was monitored at 205 nm. The hormone levels were quantified by reference to a calibration curve prepared from a serial

dilution of 90% pure GA4 (SIGMA). The GA4 retention time was 7.8 ± 0.2 min. The results were analyzed with Galaxie software, version 1.9.302.530 (VARIAN). The detection limit for GA4 was 1µg/mL.

Determination of tZR by HPLC: The procedure for the quantification of tZR was modified from the methodology originally described by Aktas *et al*. (2008), using a high-performance liquid chromatograph (VARIAN Model 920LC) with a UV detector. The tZR separation and identification were performed at room temperature using an AQUASIL C18 column with the following dimensions: 250 mm length, 4.6 mm internal diameter, and 5 µm particle size (THERMO SCIENTIFIC). The mobile phases, containing 100 mM acetic acid and 100% acetonitrile mixed 80:20 (v/v), were used in isocratic mode at 0.3 mL/min flux. The plant extract injection volume was 50 µL, and molecule detection was monitored at 268 nm. To quantify hormone levels, a calibration curve was implemented by using 95% pure tZR (SIGMA) to generate different concentrations. The tZR retention time was 15.4 ± 0.25 min. The chromatography results were analyzed with Galaxie software, version 1.9.302.530 (VARIAN). The detection limit for tZR was 0.05µg/mL.

GA4/tZR ratio estimation: The GA4/tZR ratio was the quotient obtained by dividing the GA4 concentration by that of tZR, with both concentrations obtained in the same plant organ collected during the same sampling.

Statistical analysis: The GA4 and tZR results and phytohormone ratio (GA4/tZR) data were evaluated using repeated measures ANOVA ($\alpha \leq 0.05$). This procedure was followed by Fisher's least significant difference (LSD) test ($\alpha \leq 0.05$) to compare the means that showed statistical significance in the ANOVA. The statistical tests were performed using Statistica software, version 7.0.n.

Results

The repeated measures ANOVAs for the GA4 content, tZR content, and GA4/tZR ratio (Tables 1, 2 and 3) showed significant differences for the sex, organ, and location factors and for the interaction of these three factors, except that for the tZR content, no significant difference was recorded for sex or for the interaction among the three factors.

GA4 levels in different organs from pistillate and staminate *D. cedrosanum* plants located at three locations in Coahuila, Mexico: The content of GA4 was higher in the leaves than in the other evaluated organs during stages 1 and 2 (Table 1). The difference disappeared by stage 3, during which the levels of hormone were statistically identical in the leaves and crown but higher than in the inflorescence. By stage 4, the GA4 levels were similar in the three organs. The differences in GA4 content among the organs may indicate flowering induction signals because a higher GA4 content in staminate plants was found during stages 2 and 3 after inflorescence emergence. Regarding the effect of the location factor on GA4 content, the data show that the plants in GC had the lowest levels for this hormone over the four stages.

Table 1. Mean and standard error of GA4 concentration (mg/g dry weight) in different organs of *Dasylirion cedrosanum* plants collected from three locations in Coahuila.

Factor	Stage			
	1	2	3	4
Sex				
Pi	0.0904 ± 0.02 **a**	0.1068 ± 0.02 **b**	0.1559 ± 0.03 **b**	0.2478 ± 0.05 **a**
St	0.1099 ± 0.02 **a**	0.1944 ± 0.05 **a**	0.3371 ± 0.08 **a**	0.2323 ± 0.09 **a**
Organ				
I	0.0580 ± 0.01 **b**	0.1066 ± 0.03 **b**	0.0684 ± 0.01 **b**	0.1673 ± 0.05 **a**
C	0.0747 ± 0.02 **b**	0.0918 ± 0.02 **b**	0.3090 ± 0.11 **a**	0.3335 ± 0.14 **a**
L	0.1678 ± 0.04 **a**	0.2534 ± 0.06 **a**	0.3621 ± 0.07 **a**	0.2193 ± 0.04 **a**
Location				
GC	0.0492 ± 0.02 **b**	0.0878 ± 0.03 **b**	0.0889 ± 0.02 **b**	0.0760 ± 0.03 **b**
SLC	0.0995 ± 0.02 **b**	0.1090 ± 0.01 **b**	0.3141 ± 0.10 **a**	0.3839 ± 0.13 **a**
UAAAN	0.1690 ± 0.04 **a**	0.2898 ± 0.08 **a**	0.3665 ± 0.09 **a**	0.2669 ± 0.07 **ab**
Significance	**Wilk test**			
Sex (S)	P = 0.012			
Organ (O)	P < 0.01			
Location (Lc)	P < 0.01			
S x O x Lc	P = 0.002			

The data represent the mean values ± standard error. Different letter (s) correspond to significant differences at $p \leq 0.05$ by Fisher's LSD test within each factor. (Pi) Pistillate, (St) Staminate, (I) Inflorescence, (C) Crown, (L) Leaf, (GC) General Cepeda, (SLC) San Lorenzo Canyon, (UAAAN) Universidad Autónoma Agraria Antonio Narro

Table 2. Mean and standard error of tZR concentration (mg/g dry weight) in different organs of *Dasylirion cedrosanum* plants collected from three locations in Coahuila.

Factor	Stage			
	1	2	3	4
Sex				
Pi	0.0513 ± 0.01 **a**	0.0703 ± 0.01 **a**	0.0636 ± 0.01 **a**	0.0840 ± 0.01 **a**
St	0.0624 ± 0.01 **a**	0.0734 ± 0.01 **a**	0.0733 ± 0.01 **a**	0.0842 ± 0.01 **a**
Organ				
I	0.0398 ± 0.01 **c**	0.0743 ± 0.01 **a**	0.0647 ± 0.01 **a**	0.0822 ± 0.01 **ab**
C	0.0723 ± 0.01 **a**	0.0749 ± 0.01 **a**	0.0682 ± 0.01 **a**	0.0984 ± 0.01 **a**
L	0.0583 ± 0.01 **b**	0.0664 ± 0.01 **a**	0.0726 ± 0.01 **a**	0.0717 ± 0.01 **b**
Location				
GC	0.0695 ± 0.01 **a**	0.0758 ± 0.01 **a**	0.0681 ± 0.01 **a**	0.0892 ± 0.01 **a**
SLC	0.0375 ± 0.01 **b**	0.0686 ± 0.01 **a**	0.0705 ± 0.01 **a**	0.0773 ± 0.01 **a**
UAAAN	0.0656 ± 0.01 **a**	0.0709 ± 0.01 **a**	0.0663 ± 0.01 **a**	0.0864 ± 0.01 **a**
Significance	**Wilk test**			
Sex (S)	P = 0.372			
Organ (O)	P < 0.01			
Location (Lc)	P < 0.01			
S x O x Lc	P = 0.362			

The data represent the mean values ± standard error. Different letters correspond to significant differences at p≤0.05 by Fisher's LSD test within each factor. (Pi) Pistillate, (St) Staminate, (I) Inflorescence, (C) Crown, (L) Leaf, (GC) General Cepeda, (SLC) San Lorenzo Canyon, (UAAAN) Universidad Autónoma Agraria Antonio Narro

Table 3. Average values for the GA4/tZR ratio (mg/g dry weight) quantified in the leaf, crown, and inflorescence of *Dasylirion cedrosanum* plants collected in three locations in Coahuila and analyzed during four consecutive samplings.

Factor	Stage			
	1	2	3	4
Sex				
Pi	2.0700 ± 0.33 **a**	1.8640 ± 0.41 **b**	2.8789 ± 0.55 **b**	3.2801 ± 0.65 **a**
St	2.3520 ± 0.57 **a**	3.0483 ± 0.69 **a**	6.0901 ± 1.65 **a**	4.2620 ± 1.96 **a**
Organ				
I	2.2899 ± 0.64 **ab**	1.9613 ± 0.50 **b**	1.4912 ± 0.31 **b**	2.5220 ± 0.70 **a**
C	1.2537 ± 0.26 **b**	1.3541 ± 0.23 **b**	6.2848 ± 2.27 **a**	5.2248 ± 2.95 **a**
L	3.0894 ± 0.66 **a**	4.0530 ± 1.01 **a**	5.6776 ± 1.19 **a**	3.5664 ± 0.61 **a**
Location				
GC	0.6383 ± 0.14 **b**	1.2511 ± 0.45 **b**	1.4947 ± 0.39 **b**	0.8611 ± 0.23 **b**
SLC	3.2179 ± 0.57 **a**	1.9978 ± 0.24 **b**	6.4121 ± 2.06 **a**	6.6702 ± 2.67 **a**
UAAAN	2.9655 ± 0.75 **a**	4.6739 ± 1.18 **a**	5.9008 ± 1.43 **a**	3.7856 ± 0.66 **ab**
Significance	**Wilk test**			
Sex (S)	P = 0.007			
Organ (O)	P < 0.01			
Location (Lc)	P < 0.01			
S x O x Lc	P < 0.01			

The data represent the mean values ± standard error. Different letter (s) corresponds to significant differences at p≤0.05 by Fisher's LSD test within each factor. (Pi) Pistillate, (St) Staminate, (I) Inflorescence, (C) Crown, (L) Leaf, (GC) General Cepeda, (SLC) San Lorenzo Canyon, (UAAAN) Universidad Autónoma Agraria Antonio Narro

The concentration of tZR in different organs from pistillate and staminate *D. cedrosanum* plants located at three locations in Coahuila, Mexico: The tZR content varied among the three organs during the first week of inflorescence emergence (Table 2). There was a noticeable difference in tZR levels, with crown > leaves > inflorescence. The occurrence of a difference disappeared during stages 2 and 3 but reappeared during stage 4, with crown = inflorescence > leaves. This pattern could be related to the signaling associated with flowering induction, without differences in the plant tZR levels attributable to sex. Differences in tZR concentrations among the locations were only noted during stage 1, with higher hormone content in the plant samples from GC and UAAAN than in those from SLC.

GA4/tZR ratio determination in different organs from pistillate and staminate *D. cedrosanum* plants in three locations of Coahuila, Mexico: Differences in the GA4/tZR ratio were found between the sexes during stages 2 and 3 after inflorescence emergence, in similarity with the differences found for the GA4 levels. The staminate plants showed a higher GA4/tZR ratio (Table 3) than that shown by the pistillate plants. When analyzing the different organs, the GA4/tZR ratio was observed to vary during inflorescence development, with leaves = inflorescence > crown at stage 1, leaves > inflorescence = crown during the second sampling, leaves = crown > inflorescence during the third sampling, and finally crown = leaves = inflorescence at stage 4. For the GA4/tZR ratio compared among the locations, the results showed that during most stages, with the exception of the second sampling stage, the highest levels were found in the plants at SLC and UAAAN.

Analysis of the interaction among sex, organ, and location factors for the GA4 and tZR contents and the GA4/tZR ratio: The effect of the location, organ, and sex interaction showed that at UAAAN, higher GA4 levels were found in the leaves of staminate plants during stages 1, 2, and 3. However, in SLC, higher GA4 levels were obtained from the crown of staminate plants at stages 3 and 4. Finally, in GC, higher GA4 levels were present in the inflorescence of pistillate plants during stage 4 of inflorescence development (Fig. 1).

Figure 2 shows that the highest tZR values were found in the inflorescence tissue of staminate plants during stages 2 and 4 at UAAAN. Comparing organs, the highest tZR content was observed in the crown during stages 1 and 4 of inflorescence development, showing no differences between the sexes. In GC, the highest levels of this hormone were found in the crown of staminate and pistillate plants at stage 4 of inflorescence development. Finally, in SLC, the tZR concentration was high in the inflorescence tissues of pistillate plants at stages 2, 3, and 4. The leaves were the organ showing the lowest tZR levels in all the cases.

Finally, the highest GA4/tZR ratio was found in the leaves collected from the staminate plants at UAAAN during stages 1, 2, and 3, whereas in SLC, the GA4/tZR ratio was higher in the crown of staminate plants sampled at stages 3 and 4. Still, one can observe that both the staminate and pistillate plants showed the lowest GA4/tZR ratio in GC compared with SLC and UAAAN (Fig. 3).

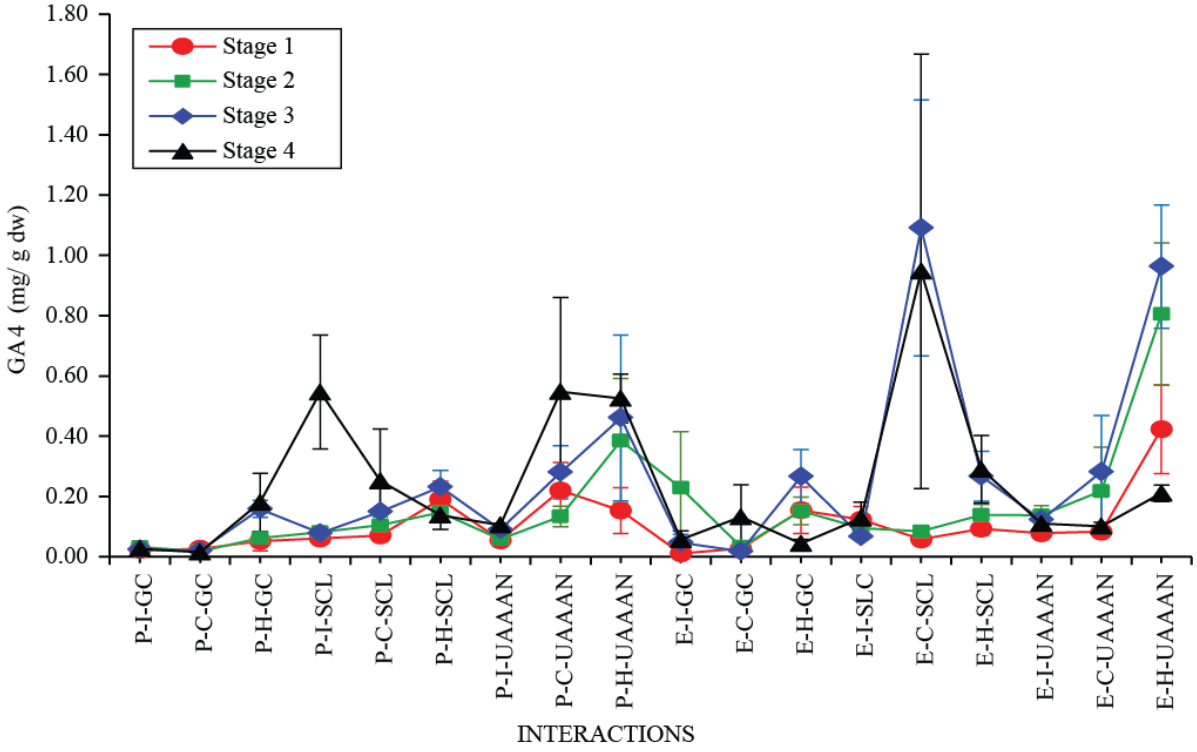

Fig. 1. Effect of sex x organ x locality interaction on the mean and standard error of the concentration of GA4 in *Dasylirion cedrosanum* plant samples collected from three locations in Coahuila. (Pi) Pistillate, (St) Staminate, (I) Inflorescence, (C) Crown, (L) Leaf, (GC) General Cepeda, (SLC) San Lorenzo Canyon, (UAAAN) Universidad Autónoma Agraria Antonio Narro.

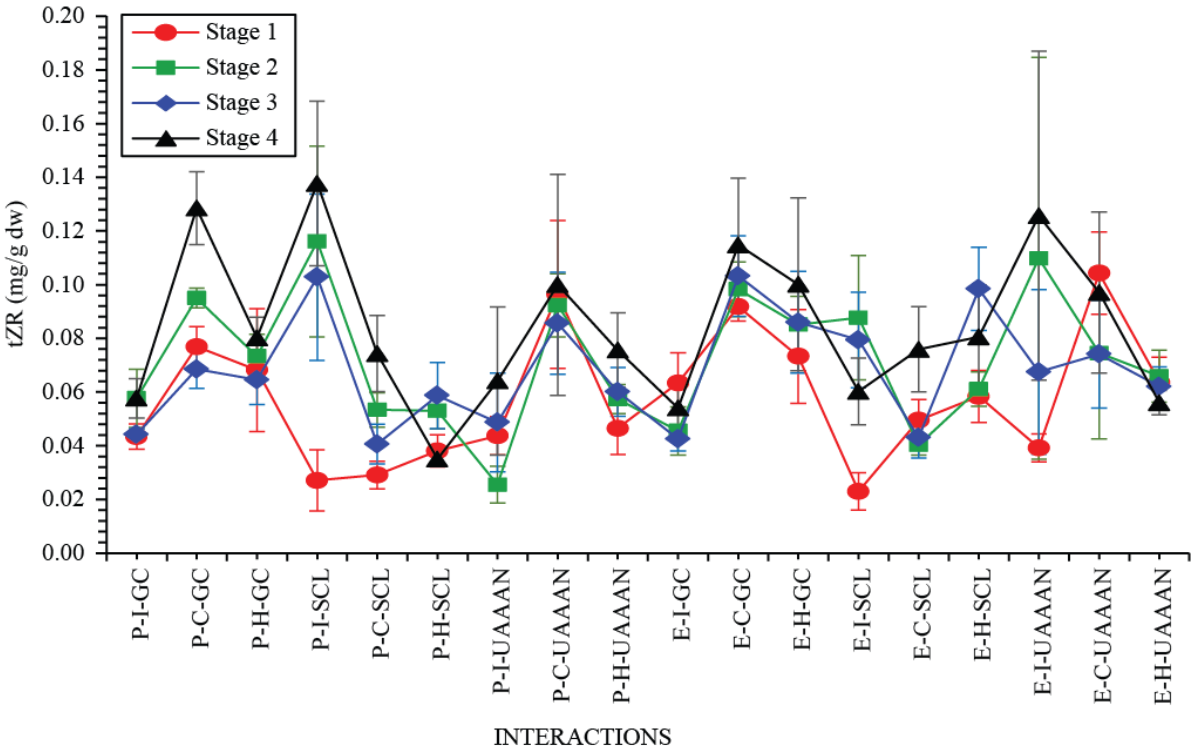

Fig. 2. Effect of sex x organ x locality interaction on the mean and standard error of the concentration of tZR in *Dasylirion cedrosanum* plant samples collected from three locations in Coahuila. (Pi) Pistillate, (St) Staminate, (I) Inflorescence, (C) Crown, (L) Leaf, (GC) General Cepeda, (SLC) San Lorenzo Canyon, (UAAAN) Universidad Autónoma Agraria Antonio Narro.

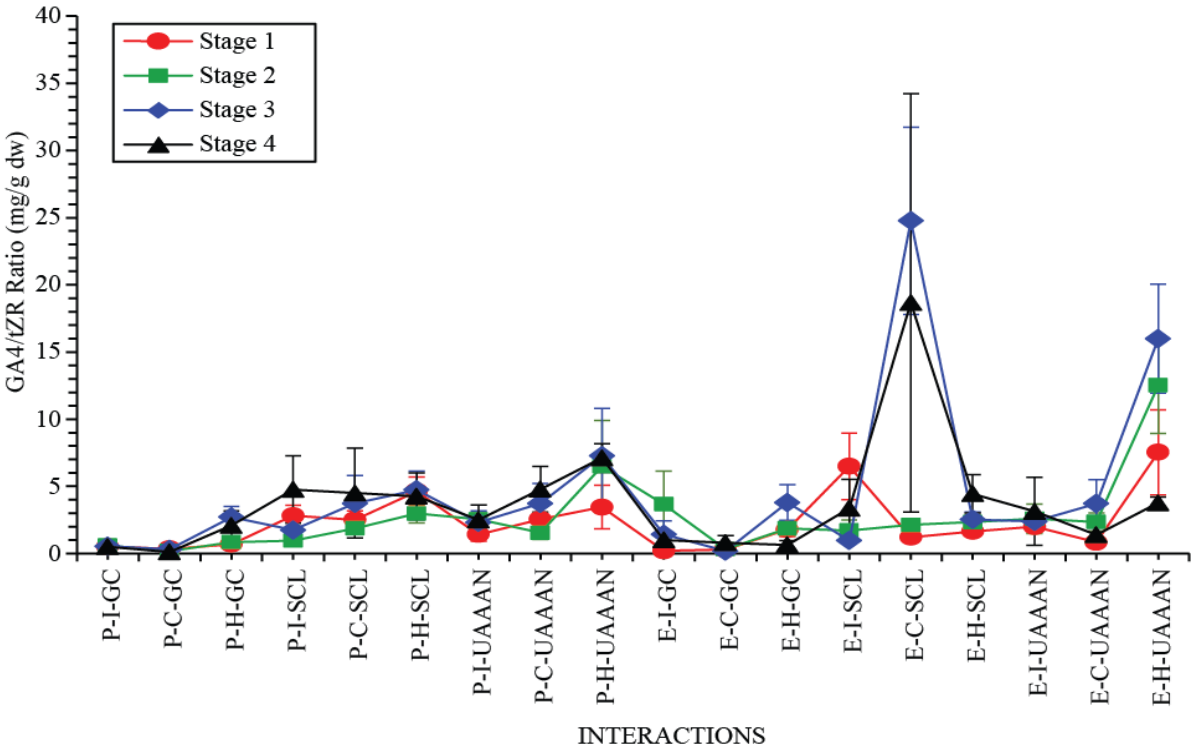

Fig. 3. Effect of sex x organ x locality interaction on the mean and standard error of the GA4/tZR ratio in *Dasylirion cedrosanum* plant samples collected from three locations in Coahuila. (Pi) Pistillate, (St) Staminate, (I) Inflorescence, (C) Crown, (L) Leaf, (GC) General Cepeda, (SLC) San Lorenzo Canyon, (UAAAN) Universidad Autónoma Agraria Antonio Narro.

Discussion

GA4 and tZR concentrations in organs of pistillate and staminate *D. cedrosanum* plants located at three sites in Coahuila, Mexico: The values in Tables 1 and 2 indicate that only the GA4 levels differed in plants of different sex and did so at stages 2 and 3 after inflorescence emergence. This result also affected the GA4/tZR ratio, which showed the highest values at the same stages. Similar results were reported by Pimenta *et al*. (2012), who analyzed endogenous levels of 7 gibberellins (GA4, GA9, GA12, GA15, GA24, GA34, and GA51) at five stages (established according to days after the emergence of the flower buds) of stamen development in male flowers of *Cucurbita maxima* L. plants. Their objective was to determine whether a relationship exists between gibberellins and the appearance of traits specific to male flowers. The results presented by these authors showed higher gibberellin levels in stamens compared with other tissues of the male flowers. In addition, the GA4 levels were higher in the stamens and other components of the male flowers after development stage 3. However, there was a generalized increase of most gibberellins in the tissues of the staminate flowers; thus, the authors pointed to gibberellins as essential for male flower development in *Cucurbita maxima*.

The effects of gibberellins and cytokinins on masculinization and feminization have also been previously reported for *Cannabis sativa*. The germination of seeds in a $Pb(NO_3)$-enriched medium led to an increase in endogenous gibberellin levels in these plants and an increased appearance of staminate plants. By contrast, the plants obtained from seeds germinated in a nutritive medium supplemented with CuSO4 and ZnSO4 showed an increase of zeatin levels, resulting in an increased frequency of pistillate plants (Soldatova & Khryanin, 2010).

Menéndez *et al*. (2006) stated that gibberellins (GAs) play a role in determining the sex of gametophytes in the fern *Blechnum spicant* L. The authors quantified the endogenous levels of GA1, GA3, GA4, GA7, GA9, and GA20 in both male and female gametophytes, producing results showing that endogenous levels of gibberellin are not significantly different between the sexes. However, the GA4, GA7, and GA20 levels were higher than those of GA1, GA3, and GA9.

In certain species such as *Chara vulgaris*, gibberellins promote the development of male sex organs, as in some ferns. However, in monocots such as corn, these hormones induce plant feminization (Vandenbussche *et al*., 2007).

The masculinizing effect of gibberellins in certain species may be explained by the results of previous studies that pinpoint the role of gibberellins in pollen viability and pollen tube development in staminate *Arabidopsis* and rice plants (Singh *et al*., 2002; Chhun *et al*., 2007). Furthermore, it is known that gibberellins play an important role during certain stages of plant development, such as the control of flowering time (Ouzounidou *et al*., 2011).

However, certain research contradicts the feminizing effect of cytokinins: Louis *et al*. (1990) reported that trans-zeatin (tZ) is related to genes responsible for feminization in *M. annua*, whereas tZR, its precursor, has been identified as an inducer of masculinization in the same species.

A number of studies show masculinization and feminization induced by gibberellins and cytokinins in plants such as *Cucumis sativus, M. annua*, *Z. mays*, and *B. dactyloides* (Yin & Quinn, 1995a, 1995b; Boissay *et al*., 1996; Zhao *et al.,* 1999). Nevertheless, this behavior is not common, as other studies show that sexual differentiation in plants is associated with other phytohormones, such as ethylene, indole acetic acid (IAA), abscisic acid, isopentenyladenosine, and dihydrozeatin riboside (Hamdi *et al*., 1987; Marziani *et al*., 1990; Kumar *et al*., 2009). All of them play an important role in sexual differentiation in plants such as asparagus (*A. officinalis*), which belongs to the *Asparagaceae* family, as does *D. cedrosanum*. In the *Asparagaceae* family, a greater concentration of abscisic acid has been observed in young staminate flowers than in young pistillate flowers (Marziani *et al*., 1990).

A study by Trebitsh *et al*. (1997) demonstrated that cucumber plants treated with a high concentration of auxins and 1-aminocyclopropane-1-carboxylic acid (ACC, an ethylene precursor) produced pistillate flowers and reduced the production of staminate flowers.

As in the above reports, we may deduce from the present results that in the case of *D. cedrosanum,* a gibberellin (GA4) plays a significant role in the appearance of staminate flowers. The tZR hormone concentrations, however, showed no differences between the plants of different sexes.

GA4/tZR ratio from pistillate and staminate *D. cedrosanum* plants located at three locations in Coahuila, Mexico: There are no reports regarding the specific GA4/tZR ratio needed to induce feminization or masculinization of plant sex organs. However, certain studies show that the relative concentration of hormones may be associated with sexual differentiation. An example of this effect can be seen in the study by Stokes *et al*. (2003), who quantified the levels of different gibberellins (GA18, GA19, GA29, and GA53) in young inflorescences of both sexes of *Rumex acetosa* L. The authors observed higher GA18 and GA29 levels in staminate plants (386.4 ± 48.8 and 363.9 ± 57.1 pmol/g dry weight, respectively) compared with pistillate plants (141.6 ± 27.2 and 163.3 ± 27.7 pmol/g dry weight, respectively). However, higher GA19 and GA53 concentrations were present in pistillate plants (538.5 ± 92.4 and 228.8 ± 22.0 pmol/g dry weight, respectively) than in staminate plants (278.3 ± 51.2 and 156.4± 23.1 pmol/g dry weight, respectively).

Liu *et al*. (2008) deduced that an increase in the relative content of tZ/IAA and GA3/tZ induces feminization of *Benincasa hispida* Cogn var. Chieh-qua plants. In addition, a higher GA3 content in stem apexes leads to the development of female flowers in this species.

In accordance with the report by Khryanin (2007) it should be mentioned that sex organ differentiation in plants is influenced by genetic, biochemical, and environmental factors. Thus, sex determination in many monoecious and dioecious species is most likely dependent on more than one of the aforementioned factors. For hormones, it would be advisable to broaden the studies distinguishing pistillate and staminate plants to assess other phytohormones together with GA4 and tZR,

including gibberellin and cytokinin isomers and conjugates, as well as to individually evaluate additional phytohormones, such as auxins, abscisic acid, and ethylene (Kumar *et al*., 2009). Furthermore, a possible relationship between the phytohormones balance should be considered as an important factor of sexual status of plants (Soldatova & Khryanin, 2010).

Analysis of the interaction of sex, organ, and location factors on GA4 and tZR content and GA4/tZR ratio: The results for the interactions among the three factors (sex, organ, and location) on the quantified hormones (GA4, tZR, and GA4/tZR ratio) showed the lowest GA4 levels for the GC location, a pattern subsequently reflected in the hormonal ratio. We are not certain why different GA4 and tZR levels were seen among the organs and stages at the different locations. One might propose that the effect of the hormones on flower induction was modified by other factors, such as irradiance, temperature, and relative humidity (Bernier *et al*., 1993) or soil mineral composition, as previously demonstrated by Sekimoto *et al*. (1997). These authors observed a decrease in GA1 levels in maize because of a deficiency in Zn. Foliar applications of $ZnSO_4$ increased GA3 and auxin levels in *Z. mays* plants (Tohidi *et al*., 2013). Similarly, Battal & Tílekliоğlu (2001) analyzed the effect of sufficient, deficient, and excessive concentrations of N, P, K, Ca, Mg, S, and Fe on tZ and tZR levels in the roots, stems, leaves, flowers, and fruits of *Z. mays* plants. The authors observed the highest tZ levels in the roots of plants treated with excess P, in the stems and female flowers of plants treated with excess K, and in the leaves and seeds of plants treated with excess Ca. The highest tZR levels, however, were observed in the roots and stems of plants treated with excess Fe and in the leaves, female flowers and seeds of plants treated with excess K.

Conclusions

GA4 varied between the sexes and among the organs evaluated in *D. cedrosanum* plants. The staminate plants presented higher levels of GA4 when compared with the pistillate plants. The variation of GA4 levels in the different organs during the stages of the sex organ development indicates a possible association of GA4 with flower induction. The tZR levels quantified in the different plant organs showed significant differences only during stages 1 and 4, which both displayed the highest level of tZR in crown tissue. The GA4/tZR ratio varied significantly between the sexes during stages 3 and 4 of inflorescence development, with the highest levels in staminate plants. For tZR, there was no significant effect of sex, even when the plants presented sex-specific morphological characteristics. The lowest GA4 levels were quantified in the staminate and pistillate plants collected from the General Cepeda location, whereas the highest levels were found in the staminate plants from SLC and UAAAN. The hormonal differences among the locations may be due to variation in the edaphoclimatic conditions existing in each region. No differences in tZR levels were detected among the locations. The behavior of the GA4/tZR ratio at the three locations reflected that of GA4.

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